# EXPRESSION OF COOPERATIVE OXYGEN BINDING AT SUBUNIT LEVEL IN EARTHWORM ERYTHROCRUORIN

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Received 28 March 1973

## 1. Introduction

Erythrocruorin is an oxygen-carrying respiratory pigment found in several species of invertebrates. It possesses the same prosthetic group as that found in vertebrate hemoglobin. However, it can be distinguished from the latter by its very high molecular weight, and by the fact that it is found dissolved in the blood fluid and not localized in blood cells. Erythrocruorin from the earthworm, *Lumbricus terrestris*, was studied by Svedberg who determined for it a molecular weight of 2.73 million and a sedimentation coefficient of 60.9 S [1]. Oxygen binding of this 60 S molecule has been shown to be cooperative by means of direct oxygen titration measurements [2]. Dissociation at alkaline pH of erythrocruorin into particles of about 10 and 3.5 S was observed by ultracentrifugation [3], and seen by electron microscopy [4]. Recent interest has arisen as to the binding behavior of the dissociated species. Wiechelman and Parkhurst [5] on the basis of fast-kinetic studies concluded that slight, if any, cooperativity can be associated with any of the alkaline dissociated products. However, inherent difficulties in their measurements precluded an unequivocal correlation of kinetic and molecular weight data. In this communication we report on the oxygen binding properties of the isolated 10 S subunit in relation to the undissociated 60 S parent molecule. Our purpose was to answer more definitely the question of whether cooperative binding extends to the 10 S subunit.

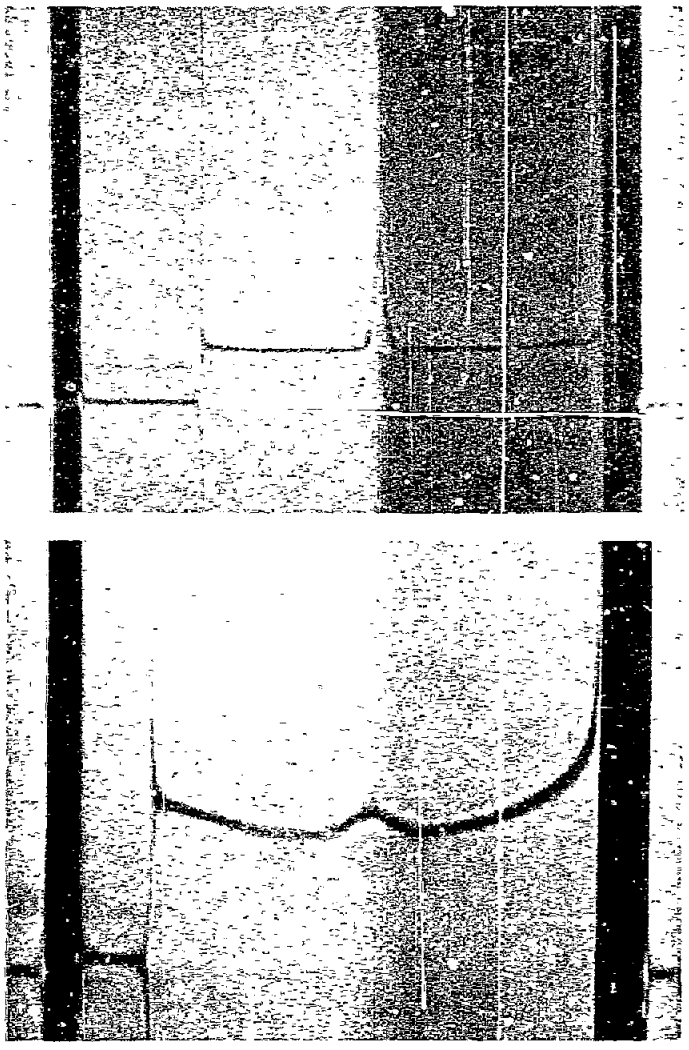

Fig. 1. Schlieren sedimentation pattern of erythrocruorin. A) Undissociated 60 S oxyerythrocruorin, 0.1 M phosphate buffer, pH = 6.8, 6 min after reaching maximum speed of 52,000 rpm. B) 10 S subunit, 0.1 M phosphate buffer, pH = 6.8, 48 min after reaching maximum speed of 56,000 rpm. Observed $s_{20,w}$ = 9.5 S.

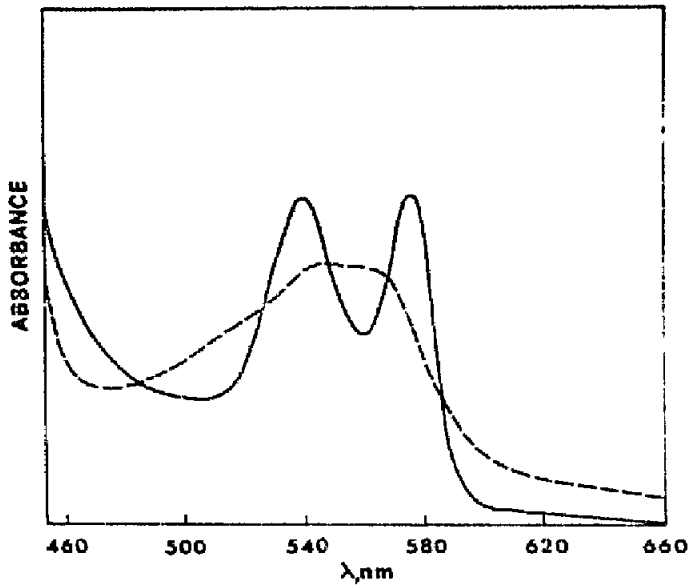


Fig 2. Absorption spectra of 10 S subunit, 0.1 M phosphate, pH = 6.8, in its oxygenated state (——), and in its deoxygenated state (----) following complete evacuation, and before commencement of titration.

## 2. Materials and methods

Erythrocruorin was prepared from locally-gathered earthworms of the family *Lumbricidae* Extraction by the method of Levin [4] yielded a preparation which sedimented as a single, sharp boundary, with $s^0_{20,w}$ = 61.1 S (fig 1A). Preparation of the 10 S subunit was carried out by incubation of undissociated oxyerythrocruorin for 24 hr in 0.1 M carbonate buffer, pH = 9 6 Following this the pH was lowered to neutrality, and isolation of the 10 S subunit effected by passage on a Sephadex G-150 column (2.7 X 60 cm) and elution by 0.1 M phosphate buffer, pH = 6.8. Fractions were concentrated by the method of ultrafiltration using a Diaflo XM-50 filter. Material prepared in this way sedimented as a single peak (fig. 1B). That the 10 S preparation was still in the oxygenated state was concluded from spectral similarity to the oxygenated 60 S molecule. Both exhibited a Soret band maximum at 414 nm, as well as two lesser maxima at 540 and 576 nm (fig. 2). Oxygen binding curves were determined spectrophotometrically by titration of deoxyerythrocruorin with oxygen. The titration vessel [6] consisted of a quartz cuvette fused to an evacuation

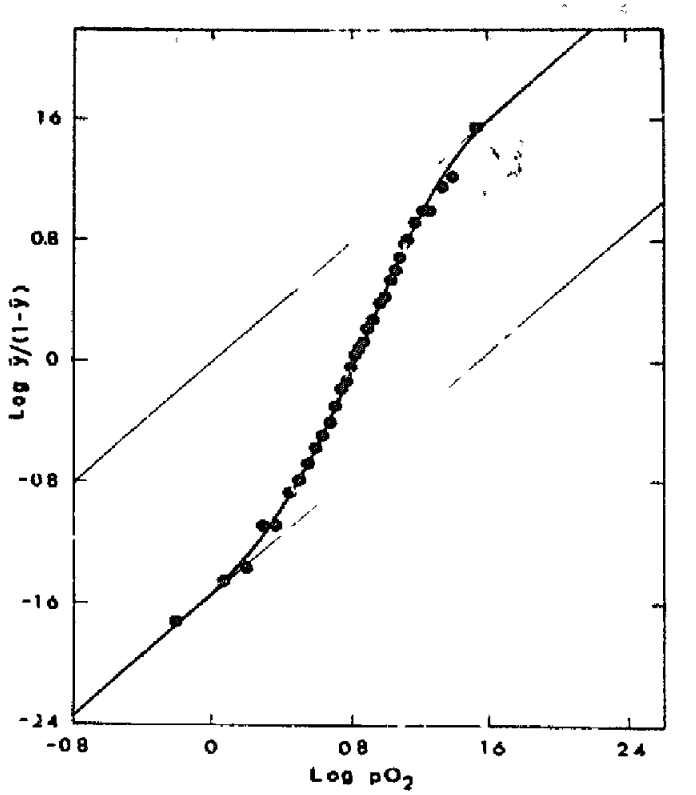


Fig. 3. Hill plot of the oxygen equilibrium of 60 S erythrocruorin, 0.1 M phosphate buffer, pH = 6.8, Temp. 26°. Concentration of protein about 1 mg/ml. Oxygen half-saturation pressure is 6.6 mm Hg, n = 2 7, and free energy of interaction is 2100 calories

chamber, at whose end were attached two stopcocks, one for evacuation and the other for introduction of measured amounts of air into the chamber The extent of oxygenation was calculated from the spectral changes at 540, 560, and 576 nm.

## 3. Results and discussion

Oxygen titration results for the 60 and 10 S molecules are presented in terms of their Hill plots (figs. 3 and 4). The slope of the curve n for a given value of $pO_2$ gives the degree of cooperativity at the corresponding saturation. Both the 60 and 10 S species show cooperative behavior as evidenced by values of n greater than unity. The minimum free energy of interaction is calculated according to Wyman [7] from the perpendicular distance between asymptotes of

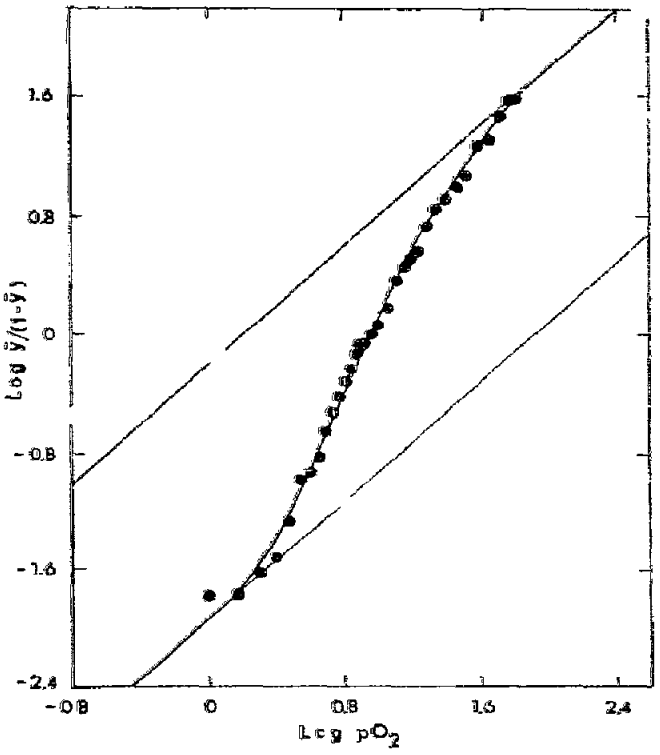


Fig. 4. Hill plot of the oxygen equilibrium of 10 S erythrocruorin. Conditions as in fig. 3. Oxygen half-saturation pressure is 8.9 mm Hg, n = 2.7, and free energy of interaction is 2300 calories.

unit slope drawn tangent to the curve at $\bar{y} \to 0$ and $\bar{y} \to 1$. Comparison of the Hill coefficients n and of the interaction energies in figs. 3 and 4 show a close similarity. The difference in the half-saturation pressures reflects a somewhat lowered oxygen affinity in the case of the 10 S subunit.

We then conclude that cooperative behavior is expressed in the 10 S subunit. The fact that the binding process in the assoc ated 60 S molecule is very similar compared to that o. the isolated 10 S subunits, implies that these subunits when assembled in the larger molecule bind independently of one another, although still binding cooperatively. Subunit assembly then appears to have little effect on the functional property of oxygen binding. What reason could then be given for the assembly of subunits in erythrocruorin? The association of 10 S subunits in the 60 S molecule, while not influencing cooperativity, is of importance in that large subunit aggregates permit a greater concentration of binding sites without an inordinate rise in osmotic pressure. Evidently, the association of non-cooperative chains to form the 10 S subunit, as in the case of alpha and beta chains in human hemoglobin, must at some stage bring about cooperativity. At what stage cooperativity begins in the assembly of the 10 S subunit, and whether the 10 S subunit is the smallest cooperative unit remains to be seen.

## References

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